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Biochemical characterization of the SecA protein of *Streptomyces lividans* Interaction with nucleotides, binding to membrane vesicles and *in vitro* translocation of proAmy protein

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The SecA protein of *Streptomyces lividans* was purified to near electrophoretic homogeneity by means of FPLC from an overproducing strain harbouring plasmid pULA400, in which the *secA* gene (Blanco, J., Coque, J. J. R. & Martín, J. F. (1996) *Gene (Amst.)* 176, 61–65) was expressed from the strong promoter of the *Streptomyces griseus saf* gene. The native form of SecA was shown to be a dimer (M_r 209 kDa) by gel filtration. It crossreacted with antibodies raised against *Escherichia coli* or *Bacillus subtilis* SecA proteins. Purified *S. lividans* SecA showed a low endogenous ATPase activity that was stimulated by addition of a *S. lividans* lipid fraction. SecA contains a high-affinity and a low-affinity nucleotide-binding site (NBS). [α -³²P]ATP could be crosslinked by ultraviolet radiation at the high-affinity site. The intrinsic tryptophan fluorescence of SecA decreased on addition of increasing concentrations of ADP and reached a saturation level at about 1 μ M (the range of saturation at the NBS I). The calculated K_d of the high-affinity binding site for ADP was 150 nM. Millimolar concentrations of ATP or ADP did not render the *S. lividans* SecA protein resistant to V8 protease degradation, in contrast to what occurs with the *E. coli* and *B. subtilis* SecA proteins. SecA was found to bind to urea-washed *S. lividans* membrane vesicles with high-affinity, i.e. 10 nM. SecA-dependent binding of *E. coli* SecB to membrane vesicles was observed when *E. coli* SecA was used, but not with the *S. lividans* SecA, suggesting that this interaction may be specific for the Gram-negative bacteria. An *in vitro* translocation system has been developed using inverted membrane vesicles of *S. lividans*. SecA supported *in vitro* translocation of proAmy into *S. lividans* membrane vesicles in an ATP-dependent manner.

Keywords: protein secretion; membrane–protein interaction; ATPase; nucleotide binding; SecA–SecB interaction.

Members of the genus *Streptomyces* are important industrial microorganisms for the production of homologous and heterologous proteins due to their ability to secrete a wide variety of enzymes and small peptides (many of them enzyme inhibitors) to the extracellular medium [1]. However, very little is known about the molecular mechanism of protein secretion in these Gram-positive bacteria. During the last few years, several genes from *Streptomyces* species homologous to *sec* genes from other bacteria, have been cloned and sequenced [2–5].

In *Escherichia coli* and *Bacillus subtilis*, the SecA protein is a dissociable subunit of the pre-protein translocase [6, 7] which plays a central role in the secretion of proteins through the cytoplasmic membrane of bacteria. SecA is able to interact with almost all the other components of the translocation apparatus [7, 8]. In *E. coli*, SecA has an ATPase activity that is activated by its interaction with translocation-competent precursor proteins, the SecY/E/G complex and acidic phospholipids [9–12]. SecA couples the binding and hydrolysis of ATP with cycles of protein

insertion and de-insertion into the membrane [13]. During membrane insertion, SecA catalyses the co-insertion of a discrete stretch of the bound pre-protein. ATP hydrolysis drives the release of the pre-protein from SecA and the de-insertion of SecA from the membrane [13, 14]. In this way, pre-proteins may be stepwise translocated through nucleotide-modulated cycles of SecA membrane insertion and de-insertion [13, 15].

It is unknown whether protein translocation in *Streptomyces* species occurs by a similar mechanism, although no SecB protein homologue has been found in Gram-positive bacteria [8]. Therefore, some differences in the protein folding and secretion mechanisms may occur in *Streptomyces* compared with *E. coli*. It was, therefore, of great interest to purify the SecA protein of *Streptomyces* and to study its interaction with ATP and the membrane. In this paper, we report the overproduction and purification of the SecA protein of *Streptomyces lividans* and its biochemical characterization. SecA was shown to support protein translocation using inverted membrane vesicles of *S. lividans*.

MATERIALS AND METHODS

Strains, plasmids and culture conditions. *E. coli* DH5 α [16] was used for plasmid isolation and for subcloning DNA fragments. *S. lividans* 1326 harbouring the multicopy pULA400

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Abbreviations. YEMES, yeast extract, malt extract, sucrose medium; NBS, nucleotide-binding site.

(which contains the *secA* gene) was used for SecA purification. *E. coli* strains were grown in Luria broth or Luria agar medium, supplemented with ampicillin (100 µg/ml) when required. *S. lividans* strains were grown in yeast extract/malt extract/sucrose (YEMES) medium (YEME+34% sucrose to allow dispersed growth) supplemented with thiostrepton (5 µg/ml) when required [17]. All plasmid constructions used derived from pUC19, pSECA1 [2] and pIJ699 [18].

Nucleic acids techniques. DNA techniques were essentially as described by Sambrook et al. [19] for *E. coli* and Hopwood et al. [17] for *S. lividans*.

SecA protein purification. *S. lividans* [pULA400] was grown at 30°C for 50 h in 400 ml YEMES medium supplemented with thiostrepton (5 µg/ml). The cells were harvested by centrifugation, washed once with TES buffer [17], suspended in 16 ml buffer A (50 mM Tris/HCl, pH 7.6; 10% glycerol and 1 mM dithiothreitol), supplemented with protease-inhibitor cocktail tablets (Boehringer, 1 tablet per 25 ml of extraction buffer) and lysed by sonication using a Branson sonicator in an ice bath. The disrupted cells were then centrifuged for 10 min at 9200×*g* and 4°C. The supernatant was ultracentrifuged at 300 000×*g* and 4°C for 30 min, filtered through a 0.45-µm Millipore membrane and then applied to a Pharmacia FPLC MonoQ 10/10 column, equilibrated in buffer A at 4°C. The column was washed with 75 ml 0.25 M NaCl in buffer A, and proteins were eluted using a 75-ml linear gradient of 0.25–0.4 M NaCl in buffer A. The *S. lividans* SecA protein eluted at 0.32 M NaCl. SecA-containing fractions were pooled, diluted ten times in buffer B (20 mM bis-Tris/propane, pH 6.5; 10% glycerol and 1 mM dithiothreitol) and applied to the same column, this time equilibrated in buffer B at 4°C. The column was washed with 75 ml 0.25 M NaCl in buffer B, and proteins were eluted with a 75-ml linear gradient of 0.25–0.4 M NaCl in buffer B. Fractions containing purified SecA protein eluted at 0.32 M NaCl. These fractions were pooled and kept at –20°C. Purity was tested by SDS/PAGE (10% acrylamide gels) after Coomassie-blue protein staining, and by immunoblotting using antisera directed against the *E. coli* and *B. subtilis* SecA.

The oligomeric nature of SecA was studied by gel-permeation chromatography using a Pharmacia FPLC Superose 6 column, equilibrated with buffer B at room temperature. Apoferritin (*M_r* 440 kDa), β amylase (*M_r* 200 kDa) and alcohol dehydrogenase (*M_r* 150 kDa) (Sigma) were used as internal standards.

ATPase assays. The ATPase activity of SecA was assayed as described by van der Wolk et al. [14]. Released orthophosphate was determined by the colorimetric assay of Lanzetta et al. [20], as described by Lill et al. [12]. For studies on lipid stimulation of SecA ATPase activity, the Mg²⁺ concentration was lowered to 0.5 mM.

Protein labelling. *S. lividans* SecA and *E. coli* SecB proteins were labelled with ¹²⁵I (Amersham) to final specific activities of about 10¹⁰ cpm/µmol, as described by Economou and Wickner [13]. Free iodine was removed by chromatography on Sephadex G25 (Pharmacia).

Photoaffinity crosslinking. Photoaffinity [α -³²P]ATP-SecA crosslinking experiments were carried out as described by Matsuyama et al. [21]. SecA (30 pmol) was incubated in 50 mM Tris/acetate, pH 7.5; 100 mM potassium acetate; 2 mM magnesium acetate and 1 mM dithiothreitol with 25 nM or 100 nM [α -³²P]ATP (3000 Ci/mmol, Amersham) for 15 min at 0°C and then subjected to photoaffinity crosslinking for 40 min at 0°C using a 254-nm lamp (model UVG-54, UVP Life Sciences Inc.) at a distance of 2 cm. Crosslinking reactions were carried out with or without ultraviolet radiation in the presence or absence of ATP at the indicated concentrations. Samples were analysed by SDS/PAGE on 10% acrylamide gels. The gels were dried and

exposed to Kodak X-Omat AR Film. Autoradiograms were scanned using a Dextra DF-2400T scanner and analysed using a SigmaScan/Imager (Jandel Corp.).

Intrinsic fluorescence measurements. *S. lividans* SecA protein at a concentration of 0.05 µM (dimer) in 50 mM HEPES, pH 7.5, 50 mM KCl, 5 mM MgCl₂ was titrated with ADP. The fluorescence emission between 320 nm and 360 nm was monitored in a SLM-Aminco 4800C spectrofluorophotometer (SLM-Aminco) with a quartz cuvette equipped with a magnetic stirrer, at an excitation wavelength of 297 nm using a bandwidth of 2 nm and a scan rate of 1 nm/s at 25°C. Spectra were corrected for the volume increase and background fluorescence.

Proteolytic digestion. SecA conformation was probed by the sensitivity to *Staphylococcus aureus* V8 protease (Sigma) according to Shinkai et al. [22]. Reaction mixtures (20 µl) contained 5 µg SecA, 50 mM Tris/HCl, pH 8.0, 50 mM KCl, 5 mM MgCl₂ and 1 mM dithiothreitol. ATPγS (1 µM or 2 mM) was added when indicated. After 10 min incubation at 37°C, 20 ng V8 protease was added, and the incubation was continued for 30 min. Reactions were stopped by adding 5 µl sample buffer supplemented with 10 mM phenylmethylsulfonyl fluoride and heating for 5 min at 95°C. Samples were analysed before and after V8 protease digestion by SDS/PAGE on 10% acrylamide gels.

Preparation of membrane vesicles from *Streptomyces lividans*. One litre of an *S. lividans* culture grown in YEMES medium supplemented with 5 mM MgCl₂ and 66 mM glycine for 48 h at 30°C was harvested by centrifugation at 8000×*g* for 10 min. Protoplasts were obtained as described by Hopwood et al. [17], centrifuged at 4000×*g* for 7 min and suspended in 15 ml Biv3 buffer (sucrose, 250 mM; Tris/HCl, pH 8.0, 50 mM; EDTA, pH 8.0, 1 mM; dithiothreitol, 1 mM). The protoplasts were lysed in a French press at 57.1 MPa. Unbroken protoplasts and protoplast debris were sedimented by centrifugation at 10 000×*g* for 10 min and the supernatant was ultracentrifuged at 200 000×*g* for 45 min. The precipitated membrane vesicles were resuspended in 300–400 µl Biv3 buffer and stored in liquid N₂. Membrane vesicles were treated with urea as described by Cunningham et al. [23].

Membrane vesicle-binding assays. SecA binding to urea-washed *Streptomyces* membrane vesicles was performed in 100-µl reactions containing 10 µg vesicles in translocation buffer [24]. [¹²⁵I]-labelled SecA was added and samples were incubated for 30 min at 0°C. Binding reactions were fractionated by centrifugation (10 min, 0.2 MPa in a Beckman airfuge, 4°C) through 100 µl 0.2 M sucrose in translocation buffer. [¹²⁵I]-proteins in pellet, supernatant and total reaction were quantified in a Beckman 8000 gamma counter. Binding data were corrected for background radioactivity determined in samples in which the membranes were excluded and plotted according to Scatchard [25].

SecA-dependent binding of *E. coli* SecB to *Streptomyces* membrane vesicles was measured by including in the reaction mixture [¹²⁵I]-labelled SecB and *E. coli* or *S. lividans* SecA proteins at 50 µg/ml.

Overexpression and purification of proAmy. A 2.3-kb *EcoRI*–*Bgl*II fragment, containing the *S. griseus amy* gene [26], was subcloned into *EcoRI*–*Bam*HI digested pGEM-3zf(+), giving rise to the recombinant plasmid pGEM3z-amy. This plasmid was mutated by site-directed mutagenesis (using the Quikchange Site-Directed Mutagenesis Kit; Stratagene) to generate a *Nde*I restriction site at the amy translation-initiation start codon. Then, the amy gene (isolated as a *Nde*I–*Hind*III fragment) was subcloned into pT7-7 vector under the control of T7 promoter, yielding plasmid pT7-amy.

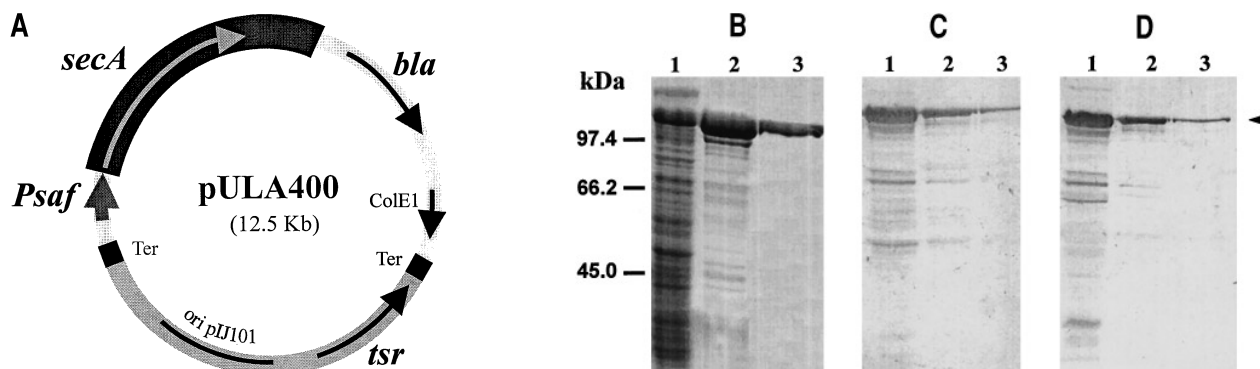


Fig. 1A–D. Plasmid construction used to overexpress the *secA* gene from the *saf* gene promoter (*Psaf*). (A) pULA400 was constructed by subcloning the *secA* gene of *S. lividans* downstream from the *saf* promoter in the positive-selection vector pIJ699. *bla*, β -lactamase gene; *tsr*, thiostrepton resistance gene; *ori*-pIJ101 corresponds to the origin of *Streptomyces* plasmid pIJ101 and *ori*-ColE1 to the origin of ColE1-derived plasmids. Ter is a transcriptional terminator. (B) SDS/PAGE of proteins in different stages of SecA purification stained with Coomassie blue. (C) and (D) Immunodetection of SecA by reaction with antibodies raised against *E. coli* SecA and *B. subtilis* SecA, respectively. Lane 1, crude extract; lane 2, fraction after first monoQ chromatography; lane 3, fraction after second monoQ chromatography. Molecular size markers (kDa) are indicated on the left.

Cells of *E. coli* BL21 (DE3) [27] transformed with pT7-amy were grown in Luria-Bertani medium at 37°C and induced with isopropylthio- β -D-galactoside (0.8 mM). After 5 h of induction, most of the synthesised proAmy protein precipitated in the form of inclusion bodies. The cells were harvested, resuspended in lysis buffer (50 mM Tris/HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA, pH 8.0; 5 mM dithiothreitol) and disrupted by sonication. Inclusion bodies (containing proAmy) were purified by standard protocols [19] and resuspended in urea buffer (50 mM Tris/HCl, pH 8.0; 6 M urea; 1 mM dithiothreitol).

In vitro translocation assay. Translocation of proAmy into urea-washed inverted *Streptomyces* membrane vesicles was assayed by its accessibility to added proteinases [9]. Reaction mixtures (50 μ l) contained 50 mM HEPES/KOH, pH 7.5; 50 mM KCl; 5 mM MgCl₂; 0.1 mg/ml BSA; 2 mM dithiothreitol; 2 mM ATP; 10 mM creatine phosphate; 50 μ g/ml creatine kinase; 50 μ g/ml SecA and membrane vesicles (20 μ g protein). Reactions were initiated by the addition of 1 μ l urea-denatured proAmy (1 μ g protein) and translocation was followed at 30°C for 20 min. Afterwards, the samples were treated with proteinase K (1 mg/ml) and/or trypsin (200 U/ml) for 15 min on ice, precipitated with 15% (mass/vol.) trichloroacetic acid, washed with acetone, and solubilised in SDS/sample buffer. Samples were separated by 10% SDS/PAGE and analysed by Western blotting with anti-Amy antisera.

Other analytical techniques. Protein concentrations were determined by the methods of Bradford or Lowry (the latter for membrane-protein quantification) using BSA as a standard. *S. lividans* lipids were isolated as described by Filgueiras and Op den Kamp [28]. Lipids were washed with acetone/ether [29] and stored at –20°C in chloroform/methanol (9:1, by vol.) under nitrogen. Protein electrophoreses were carried out in a Miniprotean apparatus (BioRad).

E. coli SecA, *E. coli* SecB and *E. coli* proOmpA were purified as described previously [14, 30]. Polyclonal antisera directed against the *B. subtilis* and *E. coli* SecA proteins were obtained from K. L. Schimz (Forschungsinstitut, Jülich, Germany) and W. Wickner (Dartmouth College, Hanover, N. H., USA), respectively. Immunoblots were developed with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) and a 5-bromo-4-chloro-3-indolyl phosphate *p*-nitro blue tetrazolium kit (Promega).

RESULTS

Purification of *S. lividans* SecA protein. *S. lividans* SecA protein was purified from an overproducing *S. lividans* strain harbouring plasmid pULA400 (Fig. 1A) in which the *secA* gene is expressed from the *saf* promoter, one of the strongest promoters known in *Streptomyces* [31, 32]. This construction was preferred to that with the native *secA* promoter because it resulted in a very high expression of the *secA* gene.

SecA was purified from extracts of *S. lividans* [pULA400] cells by using two consecutive MonoQ FPLC ion-exchange chromatography steps. A fraction from every purification step was run in a SDS/PAGE gel and analysed by Coomassie-blue staining (Fig. 1B) and by Western blotting using antibodies raised against *E. coli* SecA (Fig. 1C) or *B. subtilis* SecA (Fig. 1D). The purified *S. lividans* SecA protein crossreacted with about similar strength with both antibodies (it shares 49% and 46% identical amino acids with SecA from *B. subtilis* and *E. coli*, respectively) and migrated as a band of about 107 kDa, in good agreement with its predicted molecular mass (M_r 106 400 Da). After the second MonoQ chromatography, the protein that eluted at a NaCl concentration of 320 mM was essentially pure (on the basis of Coomassie-blue staining).

Gel-filtration chromatography on Superose 6 of the purified SecA protein using apoferritin, β amylase and alcohol dehydrogenase as internal standards showed that SecA eluted just ahead of β amylase (200 kDa) with a molecular mass of 209 kDa. This agrees with the predicted M_r of the dimer, indicating that the native SecA is a dimer, as is the case for the *E. coli* [33, 34] and *B. subtilis* [14, 35] SecA proteins.

Stimulation of SecA ATPase activity by membrane lipids. *E. coli* and *B. subtilis* SecA proteins show three different ATPase activities [11, 12, 14, 35], i.e., a low endogenous ATPase activity, a lipid-stimulated ATPase activity and the translocation ATPase activity, which requires the interaction of SecA with the precursor proteins, the SecY/E/G heterotrimeric complex and anionic phospholipids. Purified *S. lividans* SecA protein showed a low endogenous temperature-dependent ATPase activity (inset Fig. 2) which was stimulated on addition of increasing amounts of *Streptomyces* lipids (Fig. 2). No significant effect of the temperature was observed on the ATPase activity of lipid-supplemented SecA preparations. Similar observations were reported in *E. coli* by Lill et al. [12].

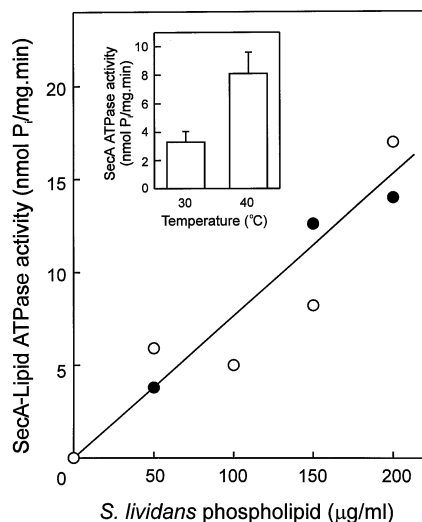


Fig. 2. Stimulation of endogenous ATPase activity of purified *S. lividans* SecA by increasing amounts of *S. lividans* phospholipids. Assays were carried out at 30°C (●) or 40°C (○). Inset, endogenous ATPase activity at 30°C and 40°C. The background ATPase level in control and phospholipid-supplemented preparations was subtracted in the main plot.

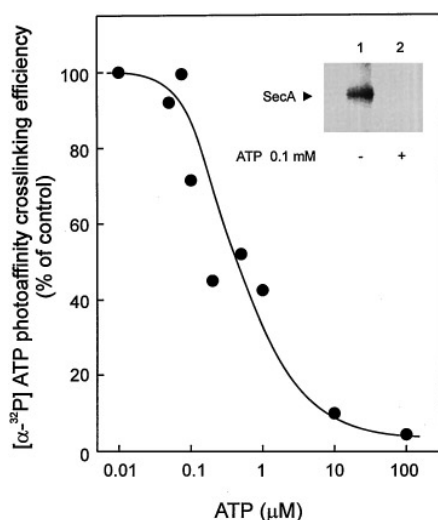


Fig. 3. Effect of increasing concentrations of ATP on photoaffinity crosslinking of SecA with $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (25 nM). Autoradiography films were densitometrically scanned, and the relative amounts of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ crosslinking of the SecA proteins were plotted as a function of the total ATP concentration. Inset: photoaffinity crosslinking of $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ with SecA as shown by SDS/PAGE of the labelled protein. Crosslinking was performed in absence (lane 1) or in presence (lane 2) of excess unlabelled ATP.

Nucleotide binding by SecA. *E. coli* and *B. subtilis* SecA proteins contain two essential ATP-binding sites: nucleotide-binding site (NBS)-I has a high affinity ($K_d \approx 150$ nM) and NBS-II has a low affinity ($K_d \approx 340$ μM) [36, 37]. Amino acid sequence analysis revealed that both NBSs are also present in the *S. lividans* SecA [2].

The *E. coli* SecA protein can be photoaffinity crosslinked at its NBS-I site with $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ at a concentration of 50–200 nM [21]. At these concentrations, hardly any crosslinking occurs at the low affinity NBS [36]. *S. lividans* SecA protein was efficiently crosslinked with 100 nM $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ upon irradiation with ultraviolet light (Fig. 3 inset, lane 1). Addition of unla-

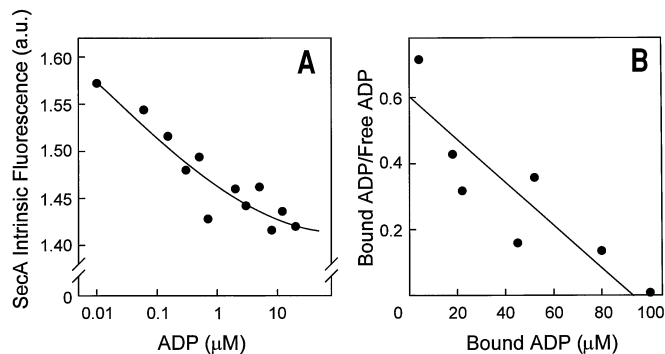


Fig. 4. Nucleotide binding at the NBS-I site of SecA as shown by changes in the intrinsic fluorescence of SecA in response to increasing concentrations of ADP (left panel). Scatchard plot of the saturation kinetics (right panel).

belled ATP to the reaction mixture prior to ultraviolet radiation prevented crosslinking (Fig. 3 inset, lane 2); about 50% inhibition was observed at 250 nM unlabelled nucleotide concentration (Fig. 3), indicating that the K_d for ATP binding to NBS-I of the *S. lividans* SecA protein was approximately 250 nM. This value is in the same range as that observed for *E. coli* SecA protein [36].

Nucleotide binding at NBS-I of SecA was further studied by analysing the changes in the intrinsic tryptophan fluorescence of the protein. *S. lividans* SecA protein has seven tryptophan residues. The fluorescence emission spectrum of the tryptophan reached a maximum at 333 nm when excited at 297 nm. *E. coli* and *B. subtilis* SecA proteins undergo large conformational changes in the presence of ATP or ADP [22, 37]. This can be monitored by following the changes in the intrinsic tryptophan fluorescence [38]. As shown in Fig. 4, the *S. lividans* SecA intrinsic fluorescence decreased on addition of increasing concentrations of ADP reaching a saturation level at about 1 μM. The observed decrease in intrinsic fluorescence occurs in an ADP range that saturates NBS-I, but does not saturate NBS-II. Therefore, assuming one ADP molecule bound per monomer in the analysis of the fluorescence data by Scatchard plot [25], an apparent K_d of 150 nM was obtained for high-affinity binding. This result is similar to the data obtained from the photoaffinity crosslinking experiment and agrees well with the K_d value determined for NBS-I of the *E. coli* and *B. subtilis* SecA proteins [36].

Binding of nucleotides at NBS-II of the *S. lividans* SecA was analysed by studying the ability of nucleotides to protect the SecA protein against *Staphylococcus aureus* V8 protease degradation. It has been reported [22, 37] that *E. coli* and *B. subtilis* SecA proteins become resistant to V8 proteolytic digestion in the presence of ATP or ADP in the millimolar range. However, in case of the *B. subtilis* SecA, if the nucleotide concentration is in the micromolar range, the protein is digested by V8. Therefore, it appears that both NBS sites need to be occupied in order to render the protein protease resistant. As shown in Fig. 5, the *S. lividans* SecA does not become protease resistant on incubation with 2 mM ATPγS (a non-hydrolysable ATP analogue) or ADP, in contrast to the protection observed with the *B. subtilis* SecA protein.

SecA binds to *Streptomyces* membrane vesicles. To analyse the binding of SecA to *S. lividans* vesicles, [^{125}I]-labelled SecA protein was mixed with urea-washed (to inactivate bound SecA) *S. lividans* vesicles and, after incubation, the membrane associated radioactivity was measured. Scatchard binding analysis (Fig. 6) indicated the existence of two components for SecA

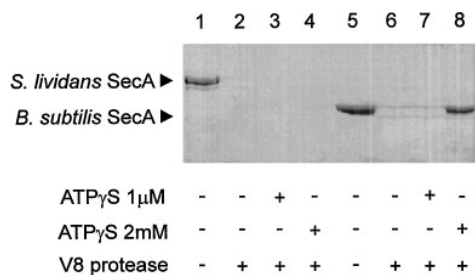


Fig. 5. Binding of nucleotides to NBS sites of SecA as shown by the ability of nucleotides to protect SecA against *Staphylococcus aureus* V8 protease degradation. The undigested SecA proteins of *S. lividans* or *B. subtilis* are shown after SDS/PAGE (10% polyacrylamide) in lane 1 and lane 5, respectively. Lanes 1–4, *S. lividans* SecA; lanes 5–8, *B. subtilis* SecA. The gel was stained with Coomassie blue. Note that 2 mM ATP is able to protect the *B. subtilis* SecA protein against V8 protease degradation (lane 8), whereas the *S. lividans* SecA was not protected by the same nucleotide concentration (lane 4).

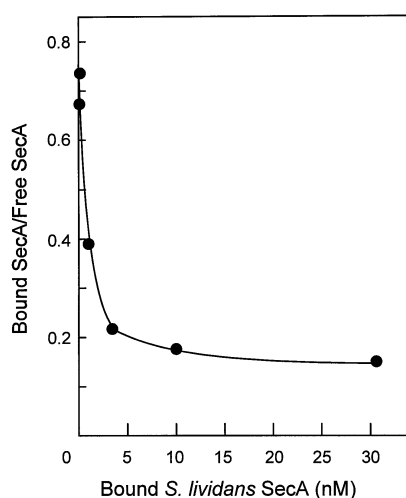


Fig. 6. Scatchard plot of binding of [125 I]-labelled SecA to urea-washed *S. lividans* membrane vesicles. Note the existence of a high-affinity and a low-affinity component in the SecA-binding kinetics.

binding: (a) a high-affinity binding site, presumably corresponding to SecY/E/G complex [24], and (b) a non-saturable component, which presumably represents association with phospholipids [39]. The calculated K_d and number of SecA binding sites was 10 nM and 40 pmol SecA/mg of membrane protein, respectively. We also studied the binding of [125 I]-labelled *E. coli* SecB to urea-washed *S. lividans* membranes. SecB is a molecular chaperone that binds the pre-protein as a nascent chain when it emerges from the ribosome [40], keeping the pre-protein in a translocation competent state [41, 42]. SecB promotes the binding of pre-proteins to SecA [24] through its interaction with the carboxy-terminal part of SecA [43]. Specific binding of SecB to *E. coli* membranes is SecA mediated.

Our results show that binding of *E. coli* SecB to urea-washed *Streptomyces* membranes depends on the origin of the SecA protein added (Fig. 7). As expected, SecB interacts with *E. coli* SecA protein, but it is not able to recognise the *S. lividans* SecA protein; rather a lower membrane binding of SecB is observed. These data suggest that the *S. lividans* SecA does not interact with the *E. coli* SecB.

The *Streptomyces* proAmy protein is translocated into inverted membrane vesicles. We have developed a homologous

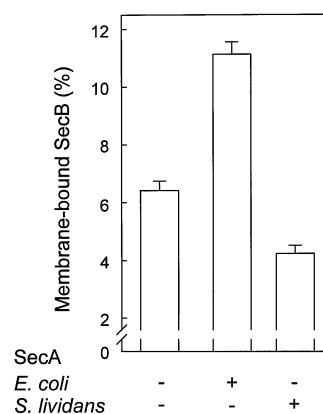


Fig. 7. SecA-dependent binding of SecB to *S. lividans* membranes. The membrane-bound amount of SecB in presence of SecA from *E. coli* or *S. lividans* is shown by vertical bars. Control reactions were performed without added SecA (left bar). The concentration of the membrane vesicles was 200 μ g/ml; [125 I]-labelled SecB was used at a final concentration of 50 nM. *E. coli* and *S. lividans* SecA proteins were used at a concentration of 50 μ g/ml.

Table 1. Stimulation of the SecA ATPase activity by proAmy in inverted membranes of *S. lividans*. Each reaction contained ATP (2 mM) in buffer C (50 mM Tris/HCl, pH 8.0; 50 mM KCl; 5 mM MgSO₄; 1 mM dithiothreitol; 0.5 mg/ml BSA), SecA (where indicated; 20 μ g/ml), proAmy (20 μ g/ml, diluted 50-fold from 1 mg/ml stock solution in urea buffer) and urea-washed *S. lividans* inverted membrane vesicles (200 μ g/ml). After incubation at 30 °C for 30 min, the inorganic phosphate liberated was quantified as described by Lanzetta et al. [20] and Lill et al. [12].

Translocation components		ATPase activity (pmol P _i /μg SecA)	
Urea-washed inverted membranes	SecA	–proAmy	+proAmy
–	–	0	250
+	–	80	600
+	+	2500	5500

translocation system using the precursor form of the *S. griseus* α amylase (proAmy) as the translocation substrate. In the presence of urea-treated *Streptomyces* membrane vesicles and proAmy, SecA displayed a significant ATP hydrolytic activity (Table 1). No translocation ATPase activity was detectable when the *S. lividans* SecA was used with *E. coli* proOmpA as the translocation substrate (data not shown).

SecA was able to support the in vitro translocation of proAmy into *S. lividans* membrane vesicles in a clearly ATP-dependent reaction (Fig. 8). In the absence of ATP and the energy regenerating system required for translocation, only a small amount of substrate remained proteinase K resistant (lane 1). This corresponds to a form of proAmy that was partially proteinase K resistant under the assay conditions (see below). The non-translocated proteinase K-resistant form of proAmy was completely eliminated upon treatment with both proteinase K and trypsin (lane 3). The presence of ATP and the energy regenerating system allowed up to 10% of the translocation substrate to remain resistant to both proteinase treatment and represent the translocated material (lane 4). No translocation of [125 I]-labelled proOmpA could be observed in this system, indicating that the *E. coli* proOmpA is not a good substrate for the *S. lividans* translocase (data not shown).

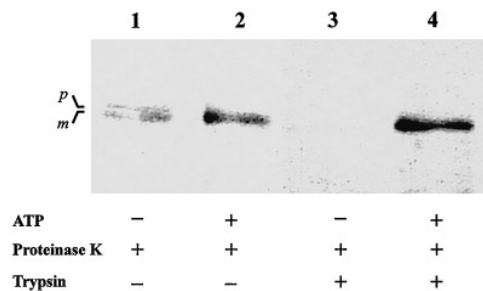


Fig. 8. *In vitro* translocation of proAmy into *S. lividans* inverted membrane vesicles is ATP dependent. After the translocation reaction (see Materials and Methods), the samples were treated with proteinase K (1 mg/ml) (lane 1 and lane 2) or a combination of proteinase K (1 mg/ml) and trypsin (200 U/ml) (lane 3 and lane 4). The protease-resistant translocated Amy was revealed with anti-amylase antibodies: p, pro-Amy; m, processed (mature) form of Amy.

DISCUSSION

S. lividans is genetically a well-known microorganism which offers great possibilities for the production of industrially important proteins and peptides since it is known to secrete large amounts of extracellular proteins [1, 32, 44, 45].

The *S. lividans* SecA protein displays biochemical features that, in many aspects, resemble those of the *E. coli* and *B. subtilis* SecA proteins. Sequence analysis of the *S. lividans* SecA indicates the presence of the two ATP-binding sites that are well conserved among the SecA proteins, i.e. the amino-terminal high-affinity NBS-I and the low-affinity NBS-II in the central region. Both NBSs are needed for activity [36]. As described in this article, the *S. lividans* SecA protein binds ATP at NBS-I with high affinity, i.e. a K_d of 150 nM. This value is in the same range as that found for *E. coli* and *B. subtilis* SecA proteins [14, 36]. With the *B. subtilis* SecA, saturation of both NBS-I and NBS-II with nucleotides results in a conformation of the protein that is resistant to *Staphylococcus aureus* V8 protease degradation [37] (Fig. 5). In contrast, the *S. lividans* SecA remains V8 protease sensitive in the presence of 2 mM ADP or ATP γ S, a nucleotide concentration that should suffice to saturate NBS-II. It appears that the *S. lividans* SecA is more readily proteolysed by V8 protease.

Purified *S. lividans* SecA shows a low endogenous ATPase activity that is stimulated by phospholipids. It was not possible to detect the 'translocation ATPase activity' using purified *E. coli* proOmpA and *S. lividans* membrane vesicles as a source of the SecY/E/G complex. ProOmpA is also a poor substrate for the *B. subtilis* SecA (van Wely, K., unpublished results). It should be emphasised that [125 I]-labelled *S. lividans* SecA binds with high affinity to urea-washed *S. lividans* membrane vesicles, suggesting that at least the binding sites (SecY/E/G complex) are preserved. The calculated K_d for SecA membrane binding is in the same range as reported for the *E. coli* SecA [46].

Although the *S. lividans* SecA binds with high affinity to the membrane, it seems that it is unable to bind the *E. coli* SecB with high affinity. The carboxyl-terminal region of the *E. coli* SecA has been implicated in SecB binding [43, 47]. Recent evidence indicates that the carboxyl-terminal 20–22 amino acids of the *E. coli* SecA are sufficient to constitute an authentic SecB-binding site [46]. With the exception of *Streptomyces*, *Mycobacterium* and *Mycoplasma* species, the carboxyl terminus of SecA is highly conserved among the bacterial SecA proteins. It may well be, therefore, that *S. lividans* lacks a SecB homologue or that it harbours some other, so far unidentified, protein with functional properties similar to the SecB protein. SecB homologues have, so far, only been found in Gram-negative bacteria.

These data, therefore, suggest that *Streptomyces* differs from Gram-negative bacteria in the targeting of pre-proteins to interact with SecA. The general secretion mechanism, however, appears to be similar.

We have developed an *in vitro* translocation system using the *Streptomyces* proAmy protein as substrate. Similarly, *in vitro* systems for translocation of precursor proteins have been developed in the Gram-positive bacteria *Staphylococcus carnosus* [48] and *B. subtilis* [49]. The yield of the *S. lividans* translocation system is still inefficient, probably due to the lack of a SecB-like component in the translocation system. This protein would putatively maintain the substrate in a translocation-competent state.

It is not known what protein(s) performs the SecB role in Gram-positive bacteria. The recent sequencing of the whole *Bacillus* genome [50] has not detected a *secB* homologue. This function may be carried out in Gram-positive bacteria by non-specific chaperones (such as GroEL). The *E. coli* GroEL has recently been shown to interact with the membrane bound SecA [51]. It, however, remains to be demonstrated whether this interaction relates to a function in protein translocation.

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